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Chlorine Resistance Patterns of Bacteria from Two Drinking Water Distribution Systems

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The relative chlorine sensitivities of bacteria isolated from chlorinated and unchlorinated drinking water distribution systems were compared by two independent methods. One method measured the toxic effect of free chlorine on bacteria, whereas the other measured the effect of combined chlorine. Bacteria from the chlorinated system were more resistant to both the combined and free forms of chlorine than those from the unchlorinated system, suggesting that there may be selection for more chlorine-tolerant microorganisms in chlorinated waters. Bacteria retained on the surfaces of 2.0-μm Nuclepore membrane filters were significantly more resistant to free chlorine compared to the total microbial population recovered on 0.2-μm membrane filters, presumably because aggregated cells or bacteria attached to suspended particulate matter exhibit more resistance than unassociated microorganisms. In accordance with this hypothesis, scanning electron microscopy of suspended particulate matter from the water samples revealed the presence of attached bacteria. The most resistant microorganisms were able to survive a 2-min exposure to 10 mg of free chlorine per liter. These included gram-positive spore-forming bacilli, actinomycetes, and some micrococci. The most sensitive bacteria were readily killed by chlorine concentrations of 1.0 mg liter^{-1} or less, and included most gram-positive micrococci, *Corynebacterium/Arthrobacter, Klebsiella, Pseudomonas/Alcaligenes, Flavobacterium/Moraxella*, and *Acinetobacter*.

Chlorination is the most widely employed method of disinfection for community water distribution systems and reservoirs. In aqueous environments, uncombined chlorine, in the form of unionized hypochlorous acid (HOCl), is an extremely potent bactericidal and virucidal agent, even at concentrations of less than 0.1 mg liter^{-1} (22). Chlorine is known to exert disruptive effects on a variety of subcellular components and metabolic processes (13), including: (i) in vitro formation of chlorinated derivatives of purine and pyrimidine nucleotide bases (10), (ii) oxidative decarboxylation of amino acids (27) and other naturally occurring carboxylic acids (19), (iii) inhibition of enzymes involved in intermediary metabolism (8, 18), (iv) inhibition of protein biosynthesis (5), (v) introduction of single- and double-stranded lesions into the bacterial chromosome (33), (vi) production of bacterial mutations (32), (vii) inhibition of membrane-mediated active transport processes and respiratory activity (8), and (viii) uncoupling of oxidative phosphorylation accompanied by leakage of macromolecules from the cell (35, 36). Chlorine has also been shown to cause physiological injury of coliform microorganisms such as *Escherichia coli*, resulting in underestimation of these indicator organisms in chlorinated waters (7, 8).

Dennis et al. (10) recently demonstrated that radioactive ^{36}Cl is preferentially incorporated into the DNA moiety of the bacteriophage f2. The uptake kinetics of free chlorine by the virus closely paralleled its sensitivity to disinfection by the halogen at different pH values. Similar findings have been reported by Haas and Engelbrecht (13) for selected bacterial cells.

Despite these and other recent advances in knowledge concerning the physiological mechanisms of chlorine disinfection, all of the physicochemical and biological parameters which influence the bactericidal properties of chlorine in the environment are not yet fully understood. Large numbers of viable microorganisms, many of which have been shown to be human secondary opportunistic pathogens, can often be recovered from potable water distribution systems maintaining free chlorine residuals (i.e., HOCl + OCl^-) of 0.5 to 1.0 mg liter^{-1} (21, 24). Thus, specific mechanisms may exist for the survival of certain bacteria and viruses in waters containing relatively high concentrations of chlorine

(14, 16). Shaffer et al. (31) recently demonstrated that poliovirus isolates obtained from fully treated, chlorinated drinking water were several orders of magnitude more resistant to free chlorine than two stock laboratory strains which were used for comparison. Furthermore, evidence has been reported which suggests that repeated exposure of poliovirus 1, strain LSc, to sublethal concentrations of chlorine may lead to physiological or genetic selection for increased resistance to this halogen (3). Some proposed mechanisms by which bacteria and viruses may develop resistance to chlorine include: (i) modification of cell surface structures which may lead to increased aggregation or clumping of cells in situ (11), (ii) microbial adhesion to pipe surfaces or to suspended particulate matter such as detritus or clay particles (34), (iii) extrusion of protective extracellular capsular or slime layers (30), and (iv) formation of resistant spores (14, 16, 17). Conceivably, some of these mechanisms may favorably influence the survival of various opportunistic pathogenic microorganisms which persist in drinking water distribution systems (21, 24).

If chlorine-resistant microbial forms exist, they should be preferentially selected for in chlorinated water distribution systems. Conversely, bacterial populations residing in unchlorinated systems should, on the whole, be skewed toward increased sensitivity to chlorine disinfection, since more resistant cell types would not possess any special selective advantage under such circumstances.

Conventional methods for determining microbial disinfection kinetics are too highly labor intensive to screen large numbers of bacterial isolates. Therefore, to determine to what extent chlorine-resistant bacteria occur in municipal drinking water supplies, two rapid screening methods were developed to compare the chlorine resistance patterns of bacteria isolated from an untreated, unchlorinated potable groundwater system and from a fully treated, chlorinated surface water system. One method (the disk assay procedure) qualitatively estimated the relative susceptibilities of individual bacterial isolates to combined chlorine. The other method (a membrane filtration procedure) measured the toxic effect of free, uncombined chlorine on bacterial populations recovered directly from the environment on the surfaces of polycarbonate Nuclepore membrane filters. Results obtained by the application of these methods to the two distribution systems investigated are described in this report.

MATERIALS AND METHODS

Description of water distribution systems. The two water distribution systems investigated were located in the cities of Irvine and Garden Grove, California. The Irvine water consists of a 60:40 blend of Colorado River water and Northern California State Project water, respectively. The water receives full treatment at the Diemer purification facility of the Metropolitan Water District of Southern California and is chlorinated to maintain a 0.5-mg liter^{-1} free residual throughout the system, which serves approximately 75,000 people.

The Garden Grove system serves a population of approximately 150,000 with groundwater drawn from 30 wells located throughout the city. The water is normally untreated and unchlorinated. However, a free chlorine residual of 0.1 to 0.2 mg liter^{-1} can occasionally be detected in certain areas of the city during the summer when some chlorinated surface water (about 10 to 20% of the total) is imported as a supplemental measure. No chlorine residual was observed in any of the Garden Grove water samples which were used for the experiments described below.

Microbiological sampling and taxonomic identification. Water samples were obtained twice monthly during the period from June 1978 through April 1980. The samples were collected aseptically from selected fire hydrants in sterile, flint-glass bottles containing 0.1 ml of 10% (wt/vol) sodium thiosulfate solution (Mallinckrodt Inc., St. Louis, Mo.). The hydrant was flushed at a flow rate of approximately 800 liters min^{-1} for 1 min immediately before sampling. The samples were stored on ice and returned to the laboratory (within 4 h) where appropriate portions (usually 0.01 to 1.0 ml) were filtered through 0.2-μm membrane filters (Gelman Instrument Co., Ann Arbor, Mich.) and placed in duplicate on *m*-Standard Plate Count medium (see ref. 24 for composition). Samples of 0.01 or 0.1 ml were also spread plated onto Plate Count Agar (Difco Laboratories, Detroit, Mich.). The plates were incubated at 35°C for 2 days at which time colonies were enumerated and transferred to Plate Count Agar slants for cold storage (4°C) and subsequent taxonomic identification. Gram-negative rod-shaped bacteria were identified to the level of genus according to a modification of the three-tube fermentation test described by Lassen (20). The Lassen method was modified by substituting Kliger Iron Agar (Difco) for the combined lactose-glucose-H_2S medium, which enhanced bacterial growth. The Lassen method was occasionally supplemented with additional biochemical tests included in the API-20 Enteric Identification System (Analytab, White Plains, N.Y.). Gram-positive microorganisms were identified according to characteristics described in *Bergey's Manual of Determinative Bacteriology, 8th ed.* (6).

Determination of bacterial chlorine resistance patterns. (i) Disk assay procedure. The disk assay technique is similar in principle and implementation to the Kirby-Bauer antibiotic disk assay method (4). In the standardized assays, bacterial isolates were inoculated into disk assay broth containing (per liter of distilled water): 0.5 g of tryptone, 0.5 g of yeast extract, and 0.5 g of dextrose (all from Difco). Cultures were incubated for 24 h at 23°C in an orbital shaker at 200 rpm. A portion of each culture was then diluted into fresh disk assay broth to a barely visible turbidity. This corresponded to an optical density (measured at 580 nm; Hitachi model 100-20 spectrophotometer) of 0.01 to 0.02 and a cell concentration (using *E. coli* strain no.

TABLE 1. Major physicochemical properties of Irvine and Garden Grove water[a]

Parameter	Unit	Irvine system		Garden Grove system	
		Source[b]	Site[c]	Source[d]	Site[e]
Free chlorine	mg liter^{-1}	0.73 ± 0.14	0.61 ± 0.15	0.005 ± 0.003	0.03 ± 0.06
Acidity	pH	7.77 ± 0.44	7.70 ± 0.58	7.22 ± 0.53	7.39 ± 0.43
Temperature	°C	17.5 ± 4.96	19.8 ± 4.72	18.9 ± 1.62	19.9 ± 2.40
Kjeldahl N	mg liter^{-1}	0.40 ± 0.09	0.17 ± 0.12	0.005 ± 0.001	0.07 ± 0.09
Ammonia N	mg liter^{-1}	0.03 ± 0.03	0.02 ± 0.02	0.02 ± 0.04	0.02 ± 0.01
Turbidity	NU	0.42 ± 0.19	2.30 ± 7.44	0.26 ± 0.11	6.61 ± 26.2
Electroconductivity	μS	836.0 ± 246.0	955.0 ± 294.0	629.0 ± 198.0	993.0 ± 335.0
Redox	mV	288.0 ± 142.0	222.0 ± 151.0	137.0 ± 43.3	97.9 ± 87.3
Dissolved O_2	mg liter^{-1}	9.69 ± 1.39	9.23 ± 1.49	7.62 ± 0.90	7.17 ± 0.99
Sodium	mg liter^{-1}	86.6 ± 3.84	85.7 ± 3.31	40.1 ± 6.77	54.8 ± 8.08
Potassium	mg liter^{-1}	4.93 ± 0.28	4.88 ± 0.35	3.86 ± 0.45	4.39 ± 0.35
Calcium	mg liter^{-1}	57.5 ± 5.43	60.0 ± 9.48	81.3 ± 9.03	89.2 ± 16.8
Magnesium	mg liter^{-1}	25.0 ± 2.36	23.3 ± 7.12	15.9 ± 3.23	21.4 ± 8.55
Manganese	μg liter^{-1}	3.50 ± 3.82	5.75 ± 4.81	3.65 ± 4.14	9.30 ± 8.56
Iron	μg liter^{-1}	26.5 ± 19.2	12.2 ± 12.3	20.0 ± 30.4	239.0 ± 326.0
Silicon	μg liter^{-1}	7.53 ± 6.27	17.7 ± 21.0	8.72 ± 10.9	11.3 ± 12.2
Cobalt	μg liter^{-1}	1.14 ± 0.86	1.19 ± 0.98	1.44 ± 1.94	1.50 ± 1.43
Copper	μg liter^{-1}	17.0 ± 45.2	6.81 ± 4.02	56.0 ± 173.0	10.6 ± 8.47
Zinc	μg liter^{-1}	4.57 ± 2.90	4.88 ± 3.76	46.1 ± 97.2	7.68 ± 7.72
Bicarbonate	mg liter^{-1}	124.0 ± 7.18	125.0 ± 7.06	215.0 ± 9.63	215.0 ± 23.7
Carbonate	mg liter^{-1}	0.66 ± 0.27	0.72 ± 0.29	0.70 ± 0.43	0.65 ± 0.26
Carbon dioxide	mg liter^{-1}	2.48 ± 1.05	2.29 ± 1.30	8.98 ± 7.58	8.25 ± 7.99
Chloride	mg liter^{-1}	84.7 ± 8.08	83.2 ± 7.26	58.2 ± 65.8	66.9 ± 10.4
Sulfate	mg liter^{-1}	206.0 ± 21.1	203.0 ± 24.6	94.2 ± 19.5	139.0 ± 33.3
Nitrate	mg liter^{-1}	0.78 ± 0.65	0.93 ± 0.68	17.6 ± 11.7	18.7 ± 8.90
P_i	μg liter^{-1}	38.0 ± 53.5	42.8 ± 58.5	40.8 ± 51.9	43.3 ± 59.6

[a] Arithmetic means ± standard deviations of bimonthly data collected from June 1978 to April 1980.
[b] Irvine source (hydrant) data.
[c] Paseo Picasso Street hydrant data.
[d] Data from well-reservoir sites 6064, 23/9, 6321, combined average.
[e] Rockinghorse and Harris Street hydrant data, combined average.

40 of our culture collection) of approximately 7.0×10^6 to 2.0×10^7 per ml. Twenty microliters of this suspension was spread plated onto the surface of disk assay agar (25 ml) contained in a plastic petri dish of standard dimensions (15 by 100 mm). Disk assay agar was prepared by adding 15.0 g of agar (Difco) per liter of disk assay broth. An environmental isolate of *E. coli* (strain no. 40) was routinely used to prepare three additional plates, with each new group of isolates tested to control internal experimental variation.

Sterile filter paper disks (7.0 mm in diameter) of Whatman no. 42 ashless paper were saturated with 8.0 μl of a solution of sodium hypochlorite (either 1,250, 2,500, or 5,000 mg liter^{-1}) in 10 mM monobasic potassium phosphate buffer (pH 7.0). The disks were placed on freshly seeded lawns of the test bacteria along with a control disk containing only the phosphate buffer. The plates were incubated in an upright position at 23°C for 48 h, and the diameters of the clear zones of growth inhibition which developed surrounding the disks were recorded.

(ii) Membrane filtration procedure. All operations described below were carried out aseptically at 23°C. Reagents were prepared with ultrapure glass-distilled water. A portion of the water distribution sample (0.05 to 200 ml, depending on the bacterial density) was filtered through a 0.2-μm polycarbonate Nuclepore membrane filter (Nuclepore Corp., Pleasanton, Calif.) held in a standard 47-mm-diameter glass Millipore filter assembly (Millipore Corp., Bedford, Mass.). The membrane surface was immediately washed with 10 ml of 5.0 mM monobasic potassium phosphate buffer (pH 7.0). After the buffer passed through the filter, the vacuum was broken and the membrane rapidly flooded with 25 ml of a 0.1-mg liter^{-1} solution of sodium hypochlorite. The chlorine solution was prepared by diluting a stock 5.0% (wt/vol) aqueous solution of sodium hypochlorite (Baker Analyzed Reagent; J. T. Backer Co., Phillipsburg, N.J.) in 5.0 mM monobasic potassium phosphate buffer (pH 7.0). The filter assembly was covered, and the chlorine solution was allowed to remain in contact with the membrane surface for 2 min. The vacuum was then reapplied, and immediately after the chlorine solution passed through the filter (approximately 15 s), the membrane was washed with 10 ml of a 10 mM solution of sodium thiosulfate in the 5.0 mM potassium phosphate buffer. The membrane was quickly removed from the filter apparatus, which was still under vacuum, and placed on the surface of *m*-Standard Plate Count medium. Duplicate membranes were prepared for several chlorine concentrations (e.g., 0.05, 0.1, 0.5, 1.0, and 10 mg of sodium hypochlorite per liter) and bacterial densities (0.05 to 200 ml). Untreated control membranes, exposed only to the phosphate buffer and thiosulfate solutions, were prepared along with the chlorine-treated membranes. The plates were incubated at 35°C, and colonies were enumerated after 2 days.

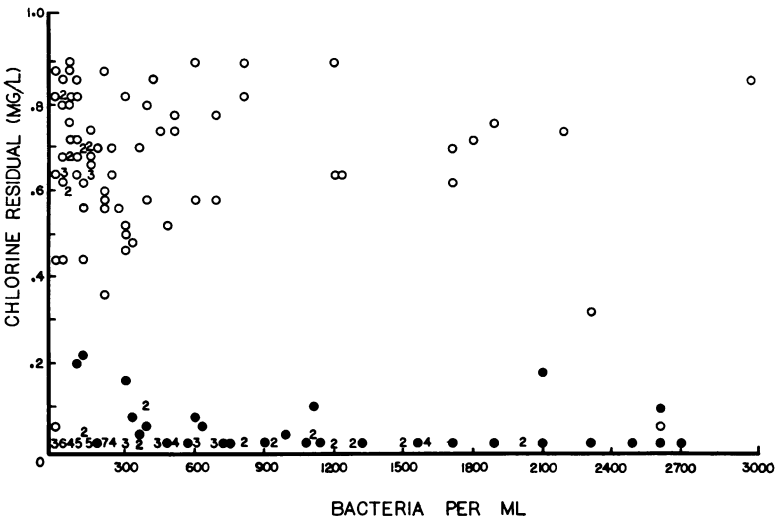

FIG. 1. Number of bacterial colonies recovered on *m*-Standard Plate Count medium as a function of the in situ concentration of free available chlorine. Bimonthly data collected from June 1978 through April 1980. Symbols: ○, Irvine data; ●, Garden Grove data.

Liquid assay procedure. To determine how the disk assay and membrane filtration procedures compared with more conventional methods of measuring chlorine disinfection, the two procedures were run in parallel with a liquid assay technique, which was performed as follows. Bacteria were grown overnight in tryptic soy broth (Difco) in an orbital shaker at 23°C. The cells were harvested by centrifugation at 3,500 × *g* for 15 min and washed twice in sterile ice-cold 50 mM monobasic potassium phosphate buffer (pH 7.0). After the final centrifugation, the bacteria were suspended in buffer to an optical density of 1.05 (measured at 580 nm), and 10 μl of this suspension were transferred to each of 12 reaction tubes containing 10 ml of the 50 mM phosphate buffer. The final cell concentration in each tube was approximately 4.0 × 10^6 per ml. The tubes were mixed thoroughly and a different amount of sodium hypochlorite was rapidly injected into each at 0 min to initiate the reaction. The last tube in the series served as an untreated control and received no chlorine. The applied sodium hypochlorite concentrations in the tubes were varied from 0 to 2.5 mg liter^{-1}. After 10 min at 23°C, 100 μl of sterile 1.0 M sodium thiosulfate was added to each tube. The tubes were chilled to 2°C, and portions were plated on Plate Count Agar to rescue surviving bacteria. The plates were incubated at 35°C for 48 h, at which time colonies were enumerated.

The effect of combined chlorine on bacteria was examined in a similar fashion. Cells were grown in disk assay broth but were diluted without prior washing to an optical density of 1.05 (at 580 nm) in fresh broth. A final concentration of dissolved organic substances of approximately 1.5 mg liter^{-1} was obtained after subsequent dilution in the 10-ml reaction tube, as described above. This concentration of organic substances was sufficient to convert all of the applied sodium hypochlorite to combined chlorine at the start of the reaction.

For kinetic studies, *E. coli* (strain no. 40) cells were grown and washed in phosphate buffer as described above and diluted into 500 ml of sterile 10 mM monobasic potassium phosphate buffer (pH 7.0), yielding a final cell concentration of approximately 1.0 × 10^7 per ml. Disinfection was initiated by adding sodium hypochlorite with continuous stirring to give a final concentration of 0.1 mg liter^{-1}. A 0.1-ml sample was removed at intervals and injected into 10 ml of ice-cold 10 mM phosphate buffer containing 10 mM sodium thiosulfate and 0.1% (wt/vol) peptone (Difco) to stop the reaction. The cells were diluted and plated on Plate Count Agar, and colonies were enumerated after incubation for 48 h at 35°C.

Chlorine demand measurements. The chlorine demand exerted by the polycarbonate Nuclepore membranes or the nitrocellulose Gelman membranes was determined by immersing a single filter (47-mm diameter; 0.2-μm pore size) in 200 ml of sodium hypochlorite solution in 5.0 mM monobasic potassium phosphate buffer (pH 7.0). Fourteen chlorine solutions ranging in concentration from 0 to 2.5 mg of sodium hypochlorite per liter were examined for each membrane type. The chlorine was reacted in the dark with the membrane for 45 min at 23°C. The free chlorine residual was then determined amperometrically by using a Fischer-Porter chlorine titrator (model 17T1010). The titrations were carried out according to the instructions of the manufacturer by using phenylarsine oxide as the reductant.

In some cases, the free available chlorine concentration remaining above the Nuclepore membrane filter surface under actual experimental conditions was monitored over a 2-min time course by the *N,N*-diethyl-*p*-phenylenediamine procedure (1a). A Hach (Hach, Ames, Iowa) model CN-66 free and total chlorine test unit was utilized for this purpose.

The chlorine demand of the disk assay broth was determined by adding increasing amounts of a 5.0% (wt/vol) sodium hypochlorite solution to a series of 15-ml samples of the medium. After a 1-h contact time,

the total residual chlorine was determined by the acidic iodine-thiosulfate procedure with starch endpoint. Alternatively, the medium was diluted 1:100, and several 200-ml samples were dosed with different concentrations of sodium hypochlorite (10 to 30 mg liter^{-1}). After a 30-min contact time, the chlorinated solutions were titrated with phenylarsine oxide at pH 7.0 for free chlorine, at pH 7.0 with added iodide for monochloramine, or at pH 4.0 with added iodide for dichloramine. The total chlorine residual was calculated by summation of the endpoints.

Physicochemical analyses of water samples. Physicochemical analyses of water samples were performed according to previously described methods (28). Field determinations of free residual chlorine were determined amperometrically.

Scanning electron microscopy. Scanning electron microscopy of suspended particulate matter in water samples was performed according to previously described procedures (29).

RESULTS

Physicochemical and bacteriological properties of distribution systems. The major physicochemical characteristics of the influent (source) water and distribution system water (from selected hydrant locations) for the cities of Irvine and Garden Grove are shown in Table 1. Both waters were of high quality according to current standards, similar in mineral composition, and of relatively low turbidity. The most significant difference between the two systems for the purposes of the present investigation was the maintenance of a free available chlorine residual (i.e., HOCl + OCl^-) of about 0.6 to 0.7 mg liter^{-1} throughout the Irvine system. Virtually no free chlorine was present in the Garden Grove system. Calculations indicated that at the pH values commonly measured in the Irvine system water, approximately 40%, or 0.24 to 0.29 mg liter^{-1}, of the measurable free available chlorine was present as unionized hypochlorous acid (HOCl), which is strongly bactericidal (26). Lower concentrations of inorganic chloramines and chlorinated derivatives of a variety of organic compounds also occur in the Irvine system, since significant quantities of ammonia nitrogen (0.02 to 0.03 mg liter^{-1}), Kjeldahl nitrogen (0.17 to 0.40 mg liter^{-1}), and total organic carbon were routinely detected (Table 1). However, because of their low concentrations and reduced toxicities compared with uncombined hypochlorous acid (22, 26), the inorganic chloramines and chlorinated organic derivatives formed should not contribute appreciably to the total bactericidal activity of the water. Inorganic ions which can potentially interfere with in situ chlorine measurements, such as the oxidized forms of manganese and iron, were present in sufficiently low concentrations as to not be problematic (Table 1).

Despite consistently high levels of free available chlorine (above 0.5 mg liter^{-1}) throughout the Irvine distribution system, large numbers of bacteria (>500 colony-forming units ml^{-1}) were regularly isolated from water samples (Fig. 1). Whereas the in situ concentration of free chlorine correlated negatively with the total number of viable bacteria (as determined by standard-plate-count procedures), the Pearson r correlation coefficient relating these parameters was not statistically significant ($r = -0.089$). Numerous instances occurred in the Irvine system in which greater than 500 bacteria per ml were recovered from water containing more than 0.6 mg of free available chlorine per liter. These data suggest that certain bacteria may possess mechanisms which enable them to survive in highly chlorinated waters.

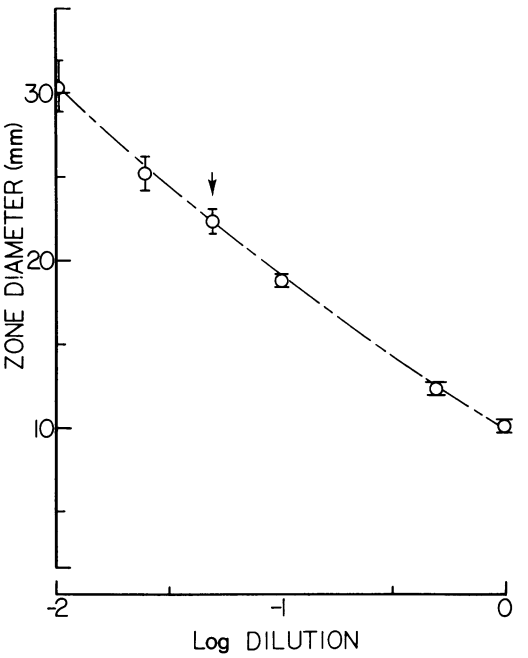

FIG. 2. Inhibition zone diameter as a function of the log of the dilution of a medium containing (per liter distilled water): glucose, 10 g; yeast extract, 10 g; tryptone, 10 g. Arrow indicates the strength of the medium used in the standard disk assay was 0.5 g each of the above components. All media contained 15.0 g of agar per liter. Each data point represents five replicates. Forty micrograms of sodium hypochlorite were applied to each disk. *E. coli* strain no. 40 cells were used.

Standardization of the disk assay. Since a number of physicochemical parameters influenced the size of the inhibition zone produced in the disk assay, it was necessary to rigorously standardize the assay conditions. The most significant factors affecting zone size were: (i) the chlorine demand of the growth medium, (ii) the

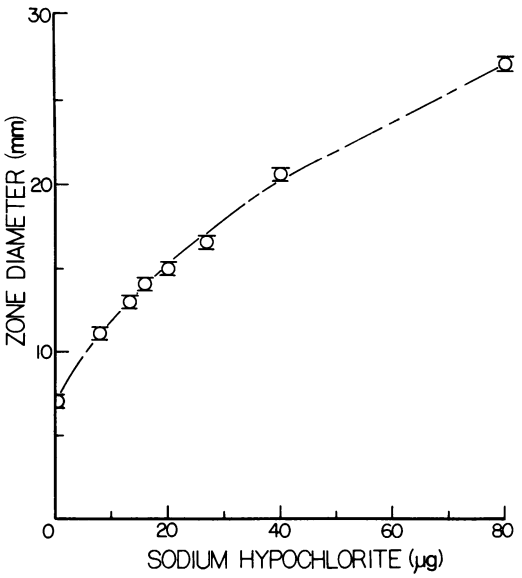

FIG. 3. Inhibition zone diameter as a function of the amount of sodium hypochlorite applied to the disk. Each data point represents four to nine replicates. *E. coli* strain no. 40 cells were used.

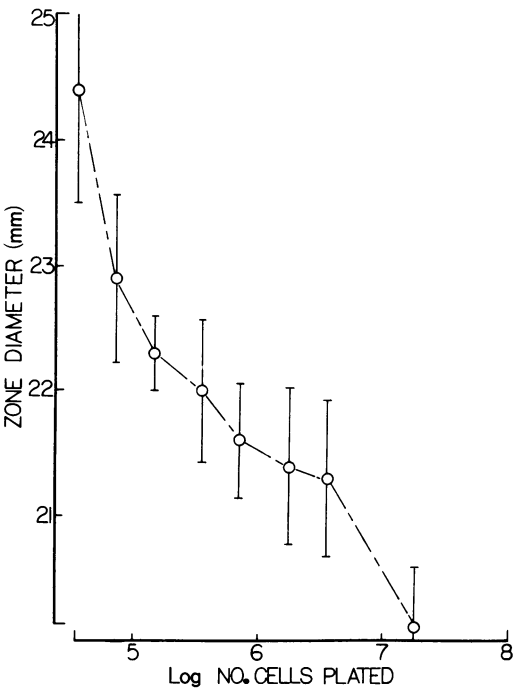

FIG. 4. Inhibition zone diameter as a function of the log of the total number of *E. coli* (strain no. 40) cells plated. Each data point represents nine replicates. Forty micrograms of sodium hypochlorite were applied to each disk.

concentration of sodium hypochlorite applied to the disk, and (iii) the number of bacterial cells applied to the surface of the medium.

The organic nitrogen concentration of the disk assay broth was approximately 213 mg of N per liter, whereas the ammonia nitrogen concentration was about 6.1 mg of N per liter. This high organic nitrogen concentration was reflected in the shape of the dose-residual (breakpoint) curve for the medium, which indicated a large combined chlorine residual. (Although in the waterworks industry the term "combined chlorine" usually refers only to inorganic monochloramine and dichloramine, we use this term in a broader sense here and elsewhere in this paper to also include halogen-substituted organic compounds.) Since the reactions of chlorine with ammonia are usually completed rapidly at pH 7.0, the persistence of a combined residual indicated the presence of relatively stable chlorinated organic nitrogen compounds (26). Though the presence of such chlorinated organic compounds precluded an accurate breakpoint determination, a markedly upward inflection occurred at about 1,900 mg of applied chlorine per liter. Since the ratio of organic to ammonia nitrogen in the medium was about 35:1, comparatively small quantities of free monochloramine were produced. Because the medium exhibited a large molar excess of chlorine demand compared to the amount of chlorine applied to the disks, it was concluded that the inhibition zones resulted from the diffusion outward from the disks of a complex mixture of inorganic chloramines and stable chlorinated organic nitrogen compounds which exhibited bactericidal activity. The specific chemical identities of these chlorine derivatives were not investigated here.

An increase in the chlorine demand of the medium led to a marked decrease in the sizes of the inhibition zones (Fig. 2), presumably because of a dilution effect of the added chlorine at the higher medium demands. Nearly all of the bacterial isolates tested grew well on a medium containing 0.5 g each of tryptone, yeast extract, and glucose per liter. Since this concentration of organic compounds was also sufficiently low to allow the formation of relatively large inhibition zones for the test organisms, it was adopted for use in the standardized disk assay procedure.

There was a nearly linear relationship between the concentration of sodium hypochlorite applied to the disks and the inhibition zone diameters for *E. coli* no. 40 (Fig. 3). Although each isolate was routinely tested at four different applied sodium hypochlorite concentrations (0, 10, 20, and 40 μg per disk), only data obtained from the disk containing the highest concentration of halogen was generally used to compare zone sizes.

Bacteria themselves readily combine with free

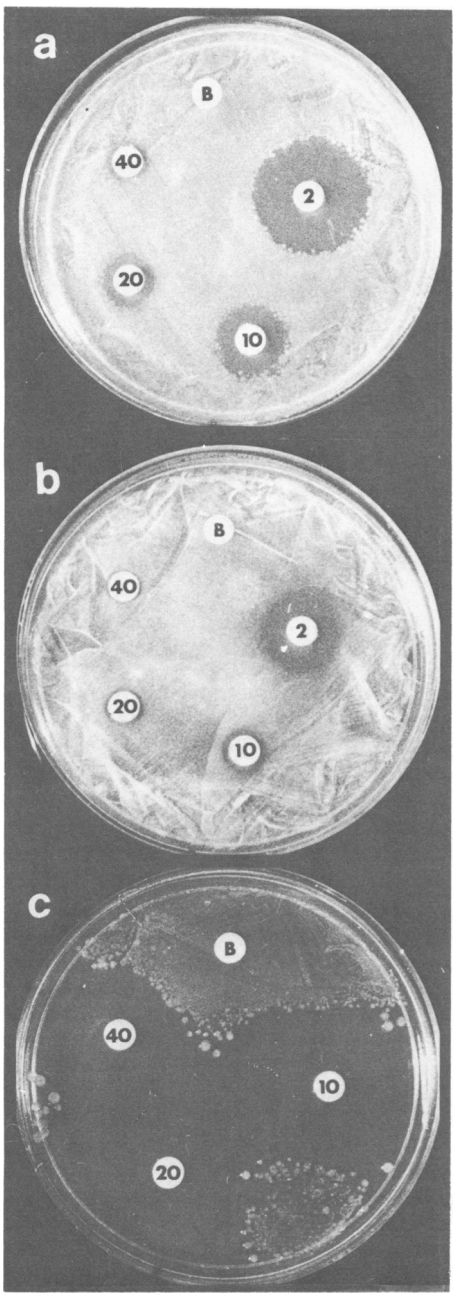

FIG. 5. Comparison of inhibition zone diameters on disk assay agar for three bacterial isolates. Plate a, *E. coli* (strain no. 40); plate b, *Acinetobacter* sp. (strain IS, 1-2) from Irvine source water; plate c, *Pseudomonas* sp. (strain 181) from Garden Grove system. Disks were saturated with: B, 8.0 μl of 10 mM potassium phosphate buffer (pH 7.0); 2, 200 μg of buffered NaOCl; 10, 40 μg of NaOCl; 20, 20 μg of NaOCl; 40, 10 μg of NaOCl. Order of sensitivity to combined chlorine: *Pseudomonas* sp. > *E. coli* > *Acinetobacter* sp.

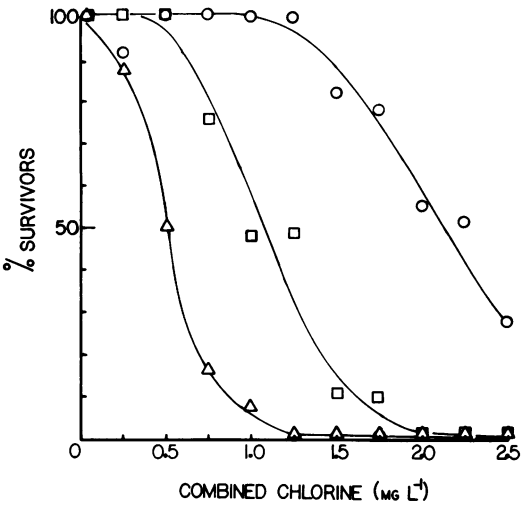

FIG. 6. Bacterial survival as a function of combined chlorine concentration in a phosphate-buffered liquid suspension. Contact time, 30 min at 23°C and pH 7.0. Symbols: △, *Pseudomonas* sp. (strain 181); □, *E. coli* (no. 40); ○, *Acinetobacter* sp. (strain IS, 1-2). Note that the relative order of sensitivity is the same as that shown in Fig. 5.

chlorine and therefore may exert a significant chlorine demand (9, 14). Thus, it was of interest to examine the effect of cell number as a function of zone size in the disk assay. These data, shown in Fig. 4, indicate that a small (4.3 mm) though noticeable increase in the mean zone diameter resulted when the number of bacteria in the inoculum was decreased from 1.9×10^7 per ml to 3.7×10^4 per ml (500-fold dilution). Therefore, before plating the bacteria, each experimental culture was routinely adjusted to a barely visible optical turbidity (optical density at 580 nm, 0.01 to 0.02). This corresponded to a cell number of approximately 7.3×10^6 to 1.8×10^7 per ml. This amount of variability in the size of the inoculum had a negligible effect on the size of the inhibition zone produced.

Each bacterial isolate tested in the disk assay exhibited a characteristic and reproducible zone size (Fig. 5). For example, the order of sensitivity to organically combined chlorine for the three organisms shown was *Pseudomonas* sp. > *E. coli* (strain no. 40) > *Acinetobacter* sp. *E. coli* consistently yielded a zone diameter of 21.8 ± 1.19 mm (n = 100) when 40 μg of sodium hypochlorite was applied to the disk. A similar order of sensitivity was observed when the above three bacteria were tested for susceptibility to combined chlorine in a buffered liquid system (Fig. 6), thereby confirming the ability of the disk technique to qualitatively estimate microbial tolerance to combined chlorine. As is the case in a liquid assay system, the bactericidal

TABLE 2. Inhibition zone diameters for bacteria from Irvine and Garden Grove systems

Bacterial group	Inhibition zone diameters (mm)[a]	
	Irvine isolates	Garden Grove isolates
Acinetobacter	22.4 ± 2.2 (51)	29.1 ± 2.4 (24)
Flavobacterium/Moraxella	18.7 ± 2.9 (60)	32.1 ± 2.0 (9)
Pseudomonas	24.0 ± 2.0 (10)	—[b]
Pseudomonas/Alcaligenes	23.6 ± 3.4 (4)	34.1 ± 8.5 (5)
Klebsiella	22.8 ± 2.0 (3)	29.1 ± 5.8 (42)
Pasturella	—	32.0 ± 0.0 (1)
Gram-positive	—	31.0 ± 0.0 (0)
Unknown	19.0 ± 1.4 (2)	24.8 ± 4.6 (2)

[a] Presented as means ± standard deviations.
[b] —, None isolated.

effect of the applied chlorine in the disk assay could be readily prevented by the addition of sodium thiosulfate to the medium.

Application of the disk assay method. The disk assay technique was applied to a total of 215 randomly selected bacterial isolates from the Irvine and Garden Grove water distribution systems. The inhibition zone diameters for the different taxonomic groups of bacteria from the two systems are shown in Table 2. In every instance, the isolates from the unchlorinated Garden Grove system exhibited larger inhibition zones, indicating that they were more susceptible to organically combined chlorine. The *Flavobacterium/Moraxella* group exhibited the largest disparity in zone size between the two distribution systems. In the Irvine system, the *Flavobacterium/Moraxella* group showed the smallest zone diameter (18.7 ± 2.9 mm), indicating it was the most resistant bacterial group, whereas the genus *Pseudomonas* was the most sensitive group (24.0 ± 2.0 mm). In the Garden Grove system, the "unknown" category of bacteria comprised the most resistant group (24.6 ± 4.6 mm), followed by *Acinetobacter* (29.1 ± 2.4 mm) and *Klebsiella* (29.1 ± 5.8 mm), respectively. The most sensitive group in the Garden Grove system was the *Pseudomonas/Alcaligenes* group (34.1 ± 8.5 mm).

A frequency histogram summarizing the zone diameters for all 215 bacterial isolates examined from both water distribution systems is shown in Fig. 7. The mean zone diameter for the Irvine bacterial isolates was 20.8 ± 3.26 mm (n = 130), whereas the mean zone diameter for the Garden Grove isolates was 30.1 ± 3.78 mm (n = 85). Though there was considerable overlap in the chlorine sensitivities of the individual isolates from the two systems, clearly the most sensitive bacteria originated from the Garden Grove system, and the most resistant microbial strains were derived from the Irvine system. Statistical analysis of these data by a standard t test indicated that the Garden Grove isolates were significantly ($P \geq 0.001$) more susceptible to organically combined chlorine than the Irvine isolates.

Standardization of the membrane filtration method. Nitrocellulose Gelman-type membrane filters, frequently employed in membrane filter enumeration techniques, were found to be unsuitable for use in the membrane filtration assay procedure described here because of an excessively high chlorine demand (approximately 1.0 mg liter^{-1}). Instead, polycarbonate Nuclepore membrane filters, which exhibited an almost

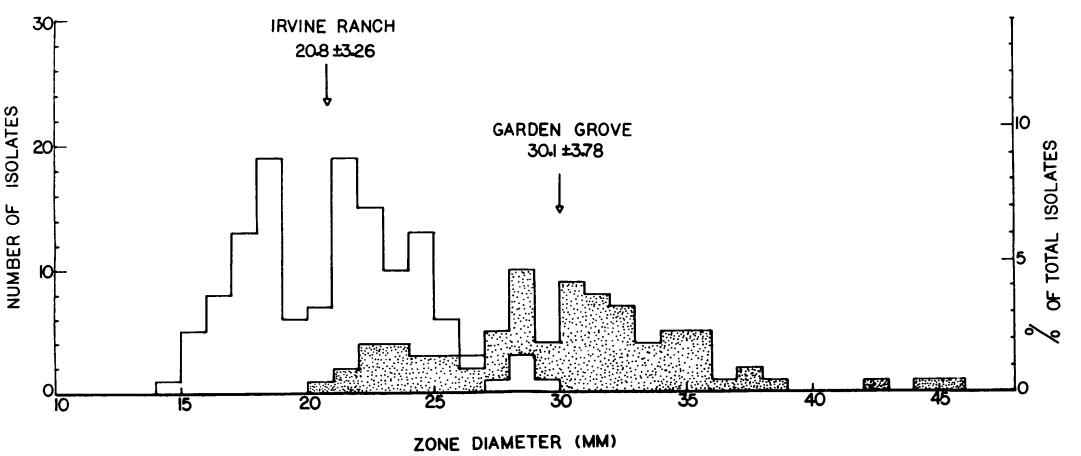

FIG. 7. Frequency histogram showing inhibition zone diameters for 130 bacterial isolates from the Irvine system (open bars) and 85 isolates from the Garden Grove system (filled bars). The mean zone diameters and standard deviations for each group are given in millimeters.

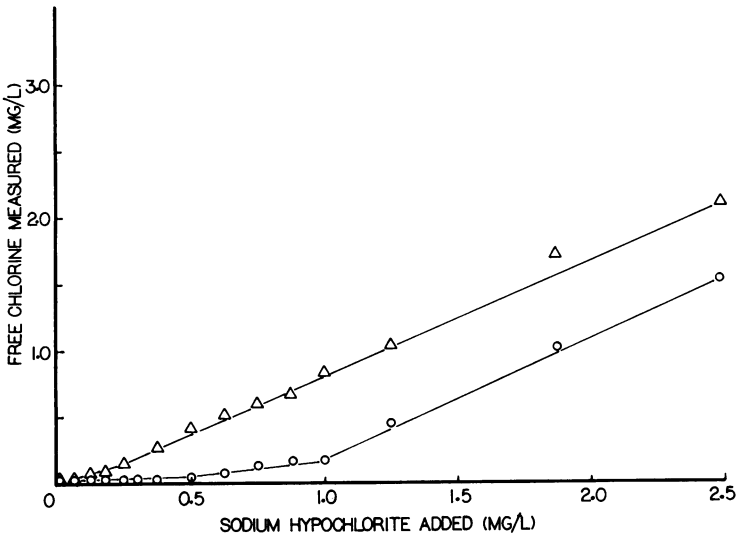

FIG. 8. Concentration of free available chlorine (determined amperometrically) as a function of the applied chlorine concentration for (△) polycarbonate Nuclepore membrane filters (0.2-μm pore size) and (○) nitrocellulose Gelman-type membrane filters (0.2-μm pore size; type 6N6). Contact time, 45 min; temperature, 23°C; pH 7.0.

negligible chlorine demand, were utilized in the assay (Fig. 8). The Nuclepore membranes possessed the additional advantages of uniform pore size and distribution and a flat surface which enhanced contact between the bacterial cells and the overlying chlorine solution.

By using the membrane filtration technique, it was possible to measure the bactericidal effects of applied chlorine concentrations as low as 0.02 mg liter^{-1} (Fig. 9). In addition, the kinetics of disinfection on the Nuclepore membrane surface closely paralleled those in buffered liquid cell suspensions during the initial 10 to 20 s of exposure to chlorine (Fig. 10). The rate of cell death on the membrane exhibited biphasic kinetics; during the initial 10 to 20 s, there was a 2 to 3 log decrease in cell viability followed by a more gradual decline. Because of the rapid nature of cell mortality, a 2-min contact time was adopted for use in the standard assay. This contact time was sufficient to attain near maximum cell kill at various applied chlorine concentrations.

Monitoring of the system amperometrically, or by using the *N,N*-diethyl-*p*-phenylenediamine procedure for detection of free chlorine, indicated that the concentration of free chlorine overlaying the membrane was essentially equivalent to the applied concentration throughout the 2-min contact period. As many as 2×10^9 bacterial cells could be filtered onto the Nuclepore membrane surface before a substantial decline was noticed in the amount of free available chlorine. Application of 2×10^9 *E. coli* cells to the membrane surface resulted in a 65.2% decrease in the free available chlorine at the end of 2 min (initial chlorine concentration, 1.2 mg liter^{-1}). In actual practice, drinking water samples always contained several orders of magnitude fewer cells than the number of cells applied in this

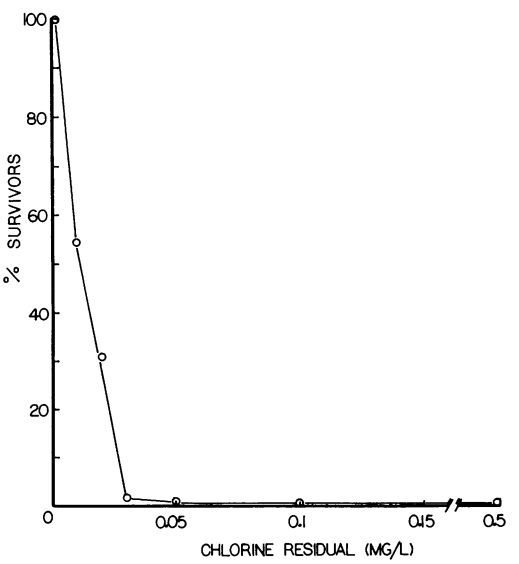

FIG. 9. Survival of *E. coli* (strain no. 40) on 0.2-μm Nuclepore membrane filters as a function of the applied chlorine concentration. Survival is expressed as a percentage of the total number of colonies appearing on untreated control membranes. Contact time, 2.0 min; temperature, 23°C; pH 7.0.

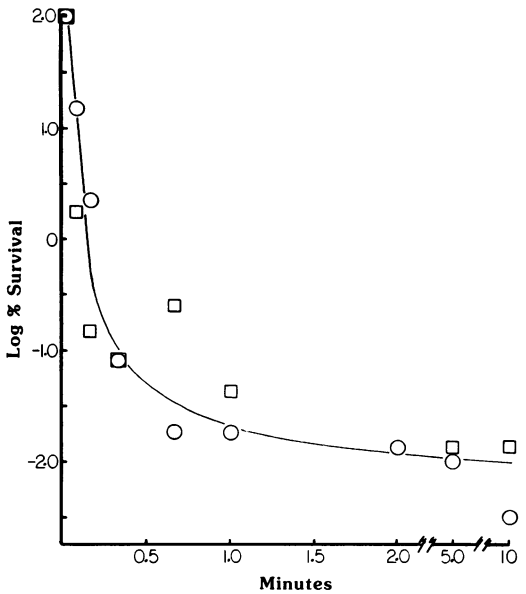

FIG. 10. Kinetics of cell death of *E. coli* on 0.2-μm Nuclepore membrane filters (□) and in 10.0 mM potassium phosphate buffer, pH 7.0 (○). The initial free chlorine concentration in both the membrane and buffer assay was 0.1 mg liter^{-1}. Temperature, 23°C. The reactions were stopped by the addition of 10.0 mM sodium thiosulfate and 0.1% peptone.

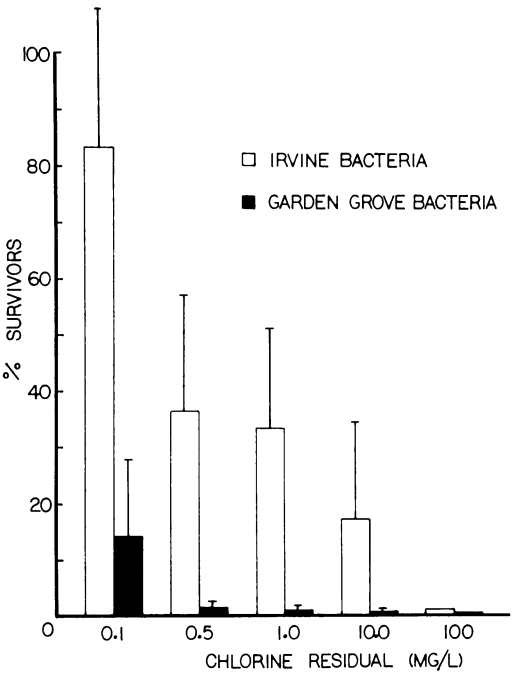

FIG. 11. Survival of Irvine and Garden Grove bacteria as a function of applied chlorine concentration by the Nuclepore membrane filtration procedure. Data shown represent mean values from six independent experiments. Irvine water samples were obtained from the source hydrant location and the Paseo Picasso Street hydrant site. Garden Grove samples were obtained from hydrants on Harris and Rockinghorse Streets. Sampling dates: 12 September 1979, 25 September 1979, 27 September 1979, 12 October 1979, 23 October 1979, and 19 January 1981. Average in situ concentration of free available chlorine for the Irvine samples, 0.82 ± 0.20 mg liter^{-1}; Garden Grove samples, 0.017 ± 0.04 mg liter^{-1}. Contact time, 2.0 min; temperature, 23°C; pH 7.0.

experiment. Thus, under experimental conditions the contribution by the cells to the total chlorine demand was insignificant. In addition, the level of bacterial survival was evidently independent of the number of cells applied to the surface of the membrane filter, unless the numbers applied were sufficiently high to result in extensive clumping.

Application of membrane filtration method. Data from six separate experiments comparing the relative susceptibilities to free chlorine of total bacteria recovered from the Irvine and Garden Grove systems are summarized in Fig. 11. The Irvine bacteria were found to be significantly more resistant to chlorine disinfection at all concentrations of halogen tested between 0.1 and 10 mg liter^{-1}. Although the Irvine bacteria were generally less numerous in situ than the Garden Grove bacteria (Fig. 1; ref. 24), they exhibited up to a sixfold-better survival when exposed to a 0.1-mg liter^{-1} solution of sodium hypochlorite. This difference in population sensitivity increased at the higher chlorine residuals utilized (e.g., 18-fold at 0.5 mg liter^{-1} and 25-fold at 1.0 mg liter^{-1}) but was virtually absent at a concentration of 100 mg liter^{-1}. Therefore, the Irvine system, which was consistently maintained at 0.6 to 0.7 mg of free chlorine per liter, harbored a microbial population significantly more resistant to chlorine disinfection than was the population of the Garden Grove system. Interestingly, however, approximately 0.5% of the total bacteria encountered in both distribution systems were extremely resistant to chlorine. These bacteria were able to survive an exposure to 100 mg of applied sodium hypochlorite per liter for the duration of the 2-min contact period.

To determine which bacterial genera were most sensitive or resistant to chlorine disinfection, a total of 260 bacterial isolates from one experiment were identified after exposure to various concentrations of free chlorine in the membrane assay. The results (Table 3) indicated that different microbial genera were killed by different levels of chlorination in the membrane filtration procedure. In the Garden Grove system, for example, the following groups of bacteria were readily eliminated from the bacterial

TABLE 3. Percentage of bacteria surviving exposure to free chlorine in membrane filtration assay procedure

Bacterial genus/taxonomic group	% Bacteria surviving chlorine concentration (mg liter^{-1}) of[a]:				
	0	0.1	0.5	1.0	10.0
Acinetobacter	21.9	36.4	—[b]	3.3	—
	—	—	(14.3)	—	—
Flavobacterium/Moraxella	—	—	—	—	—
	—	2.3	—	—	—
Corynebacteria/Arthrobacter	56.1	36.4	10.0	—	—
	—	—	—	—	—
Pseudomonas/Alcaligenes	17.1	10.7	—	—	—
	(3.0)	—	—	—	—
Klebsiella	—	2.3	—	—	—
	—	(3.6)	—	—	—
Micrococcus	2.4	4.5	—	—	—
	(84.8)	—	—	—	(50.0)
Bacillus	—	4.5	2.3	40.0	48.3
	(12.1)	(10.7)	(85.7)	(100.0)	(50.0)
Actinomycete	2.4	13.6	66.7	56.7	51.7
	—	—	—	—	—
Unknown	—	—	—	—	—
		(3.6)			
Number of bacteria identified	41	44	30	30	29
	(33)	(28)	(7)	(14)	(4)

[a] Numbers in parentheses indicate Irvine data; other numbers indicate Garden Grove data. Irvine water sample was from Paseo Picasso Street hydrant location. Garden Grove water sample was from Harris Street hydrant location. Sampling date, 19 January 1981.

[b] —, None detected.

population at applied halogen concentrations of less than 0.5 mg liter^{-1}: *Flavobacteria/Moraxella, Pseudomonas/Alcaligenes, Klebsiella*, and *Micrococcus*. The *Corynebacterium/Arthrobacter* group still exhibited about 10% survival at a free chlorine concentration of 0.5 mg liter^{-1}, but was completely eliminated at a concentration of 1.0 mg liter^{-1}. The genus *Acinetobacter*, on the other hand, exhibited a small percentage of survival (3.3%) even at a chlorine concentration of 1.0 mg liter^{-1}. Gram-positive spore-forming bacilli and actinomycetes were clearly the most chlorine-resistant cell types in the Garden Grove system, comprising 48.3% and 51.7%, respectively, of the total survivors after exposure to 10 mg of free chlorine per liter for 2 min. In the Irvine system, bacteria unable to survive free chlorine concentrations greater than 0.5 mg liter^{-1} included *Acinetobacter, Pseudomonas/Alcaligenes, Klebsiella*, and the vast majority of the gram-positive micrococci. *Bacillus* spp. were the predominant chlorine-resistant forms in the Irvine system.

Mechanism of chlorine resistance. One mechanism for bacterial resistance to chlorination is cellular aggregation or adhesion to suspended particulate matter (16). To evaluate the significance of this survival mechanism in potable water systems, the membrane filtration assay was carried out with 0.2 and 2.0-μm Nuclepore membranes. Scanning electron microscopy per-

TABLE 4. Bacterial survival on 0.2- and 2.0-μm Nuclepore membrane filters

Membrane pore size (μm)	% Bacterial survival on[a]:	
	Control membrane (no treatment)	Experimental membrane (10 mg of NaOCl per liter)[b]
0.2	100	0.74 ± 0.71
2.0	100	9.90 ± 3.93

[a] Means ± standard deviations from four separate experiments with the Harris Street (Garden Grove) water sample. Sampling dates: 2 April 1980, 8 April 1980, 18 April 1980, 19 January 1981.

[b] Contact time, 2 min.

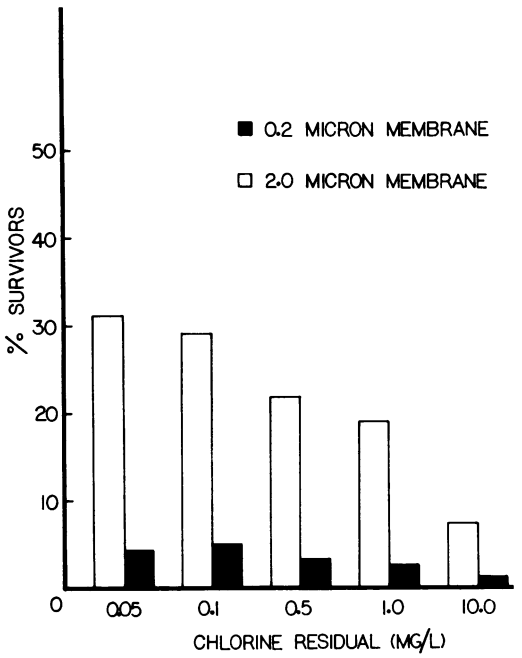

FIG. 12. Survival of bacteria from Garden Grove system (Harris Street) on 0.2- and 2.0-μm Nuclepore membranes as a function of the concentration of applied chlorine. Contact time, 2.0 min; temperature, 23°C; pH 7.0.

formed in this laboratory, as well as differential size filtration experiments done by others using radiolabeled cells (2), demonstrated that single bacteria in environmental samples are usually less than 1.0 μm in diameter and readily pass through Nuclepore membrane filters with pore diameters greater than 2.0 μm. Bacteria which are aggregated together or attached to the surfaces of suspended particulate matter should be preferentially retained by larger-pore-size filters; hence, a greater proportion of cells captured on the 2.0-μm membranes may survive exposure to higher chlorine concentrations.

The results of four independent experiments involving differential-size filtration of bacteria from the Garden Grove system are shown in Table 4. At a concentration of 10 mg of applied chlorine per liter, bacteria retained on the 2.0-μm membrane filters exhibited >10-fold-better survival than did the total microbial population recovered on the 0.2-μm filters. In each of the above cases, the number of colonies arising on the chlorine-treated membrane filter were compared with the number appearing on an untreated control filter of the same pore size. Similar data are presented in Fig. 12 for one Garden Grove water sample treated with a range of applied chlorine concentrations (0.05 to 10 mg liter^{-1}). At all concentrations of halogen tested, the bacteria retained on the 2.0-μm filters showed significantly greater survival than those on the 0.2-μm filters, suggesting that unassociated bacteria are more sensitive than aggregated or attached cells. In addition, there was an increase in the number of actinomycete colonies on the 2.0-μm control and chlorine-treated membrane filters, compared to the 0.2-μm filters. Of the bacteria which grew on the treated 2.0-μm membranes, from 30 to 60% corresponded to actinomycete colonies. Presumably, this enrichment was due to two factors: (i) the filamentous nature of actinomycetes, which would tend to impede their passage through a 2.0-μm membrane filter, and (ii) the formation of chlorine-resistant actinospores (17).

Scanning electron microscopy. The higher chlorine resistance exhibited by bacteria retained on the 2.0-μm Nuclepore membrane filters prompted a microscopic search for aggregated or attached cells. Scanning electron microscopy of suspended particulate matter recovered from the same water samples as used in the differential-size filtration experiments described above revealed bacterially colonized particles, as well as evidence of cellular aggregation (Fig. 13). Approximately 1% of the particles examined bore attached bacterial cells, which were generally rod shaped and sometimes partially obstructed from view by extracellular slime or capsular material. The number of bacteria affixed to a single particle varied from as few as 5 or 10 to as many as several hundred. Particles with attached bacteria were usually larger than about 10 μm, so they would almost certainly be captured on the 2.0-μm membrane filters. In addition to the colonized particles, branching filamentous microorganisms identical in morphology to actinomycetes were also frequently observed on the membrane filter surfaces. Although a higher resistance to chlorine disinfection was not directly demonstrated for the attached cells and actinomycete-like filaments observed in the scanning electron microscope, their preferential retention on the surfaces of 2.0-μm membrane filters is consistent with such a hypothesis.

DISCUSSION

Conventional methods for measuring microbial disinfection kinetics of free or combined chlorine often involve subculturing of bacterial isolates on artificial growth media, followed by extensive washing of the cells. These processes can lead to alteration of cell surface characteristics, and dramatic changes in the biochemical and molecular properties of the bacterial cell surface resulting from such washing procedures may significantly influence microbial disinfection kinetics. The membrane filtration procedure

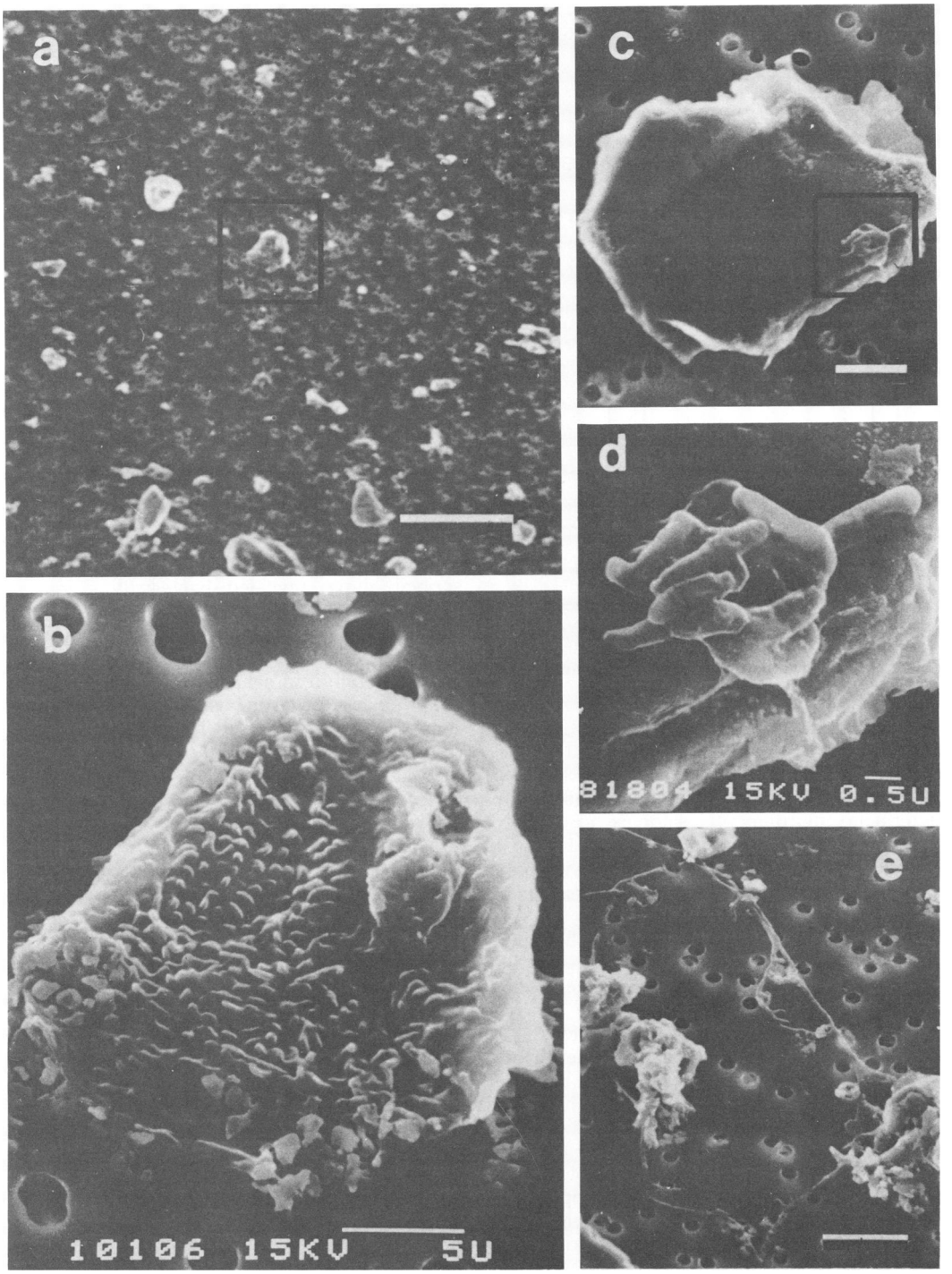

FIG. 13. Scanning electron micrographs of bacteria-laden suspended particulate matter and actinomycete-like filaments from Harris Street hydrant location (8 April 1980 water sample). (a) Low-magnification view of particles captured on surface of 2.0-μm Nuclepore membrane (bar, 50 μm). (b) Enlargement of particle delineated in (a), showing possible attached bacterial cells partially coated by extracellular slime (bar, 5.0 μm). (c) Suspended particle bearing a small clump of aggregated rod-shaped bacteria (bar, 5.0 μm). (d) High-magnification view of clump of cells depicted in (c) (bar, 0.5 μm). (e) Branching actinomycete-like filament, one end of which is partially sequestered by clump of debris (bar, 10 μm).

described in this report allows rapid and quantitative assessment of the chlorine resistance patterns of bacterial populations obtained directly from an aquatic environment soon after sampling, as opposed to testing of individual isolates after repeated subculture on artificial growth media. Possible physiological or genetic alterations of the bacterial cell wall or cytoplasmic membrane, which might result from subculturing or prolonged cold storage of isolates, are thereby precluded. Small quantities of adsorbed organic nitrogen-containing compounds, free ammonia, or other contaminating substances in the water supply which may introduce extraneous chlorine demand can be readily eliminated from the membrane assay by briefly rinsing the cells directly on the Nuclepore membrane surface immediately before chlorine exposure. In this way the potential deleterious effects inherent in extensive washing procedures are minimized.

In addition to being ideally suited for rapid screening of the chlorine resistances of large numbers of microbial isolates, the membrane filtration procedure also facilitates the detection, quantitative enumeration, and axenic isolation of highly chlorine-resistant or -sensitive cell types which may be present in a particular water supply. These bacteria may then be utilized in subsequent studies designed to elucidate the molecular and ultrastructural bases for extreme sensitivity or resistance to chlorine.

The disk assay method, which measures the relative toxicity of combined chlorine, can also be performed rapidly and inexpensively, although quantification procedures are more difficult. In addition, growth of the individual bacteria on artificial media is required. The breakpoint chlorination characteristics of the disk assay broth indicate that all of the applied chlorine which diffuses from the disk undergoes reaction with ammonia and organic nitrogen compounds in the medium (26). The large chlorine demand displayed by the medium is due principally to amino acids, short-chain polypeptides, nucleic acids, and proteins which constitute the tryptone and yeast extract components. The glucose, as well as the agar in the solid medium, would also exert a significant chlorine demand. These substances are present in the medium in large molar excesses compared to the amount of chlorine applied to the disks and are known to be extremely reactive with aqueous hypochlorous acid at neutral pH values (26, 27). Therefore, formation of a wide variety of halogen-substituted organic compounds would be expected. Such compounds apparently diffuse outward from the vicinity of the disk, establishing a concentration gradient. A minimum inhibitory concentration of these substances that is characteristic for each bacterial isolate is eventually attained at some distance from the disk. Hence, the disk assay technique represents a qualitative determination of the relative susceptibilities of individual bacterial isolates to a complex toxic mixture of chloramines and diffusible halogenated organic compounds. As such, the disk assay method may be most suitable for comparing the sensitivities of microorganisms isolated from chlorinated sewage or other wastewater sources which contain high concentrations of organic compounds and ammonia. Microorganisms which are involved in biofouling of water distribution pipe surfaces or in attachment to suspended particulate matter (28) may also be exposed to relatively high concentrations of chloramines and chlorinated organic compounds. Bacteria associated with these surface biofilms are frequently embedded within a complex organic matrix which can react with free chlorine to form a wide variety of organic chlorine derivatives (9). Therefore, the high combined chlorine residuals present in the disk assay procedure may mimic conditions found in sewage or attached biofilms containing high concentrations of organics.

The fact that relatively high numbers of microorganisms could be routinely isolated from the chlorinated Irvine system water suggests that certain bacteria may possess mechanisms enabling them to survive in highly chlorinated environments. A number of other conclusions may be inferred from the data obtained from the application of the disk assay and membrane filtration procedures, as described in this report. These conclusions are as follows. (i) Bacteria residing in the chlorinated Irvine water distribution system were, as a group, significantly more resistant to both free and organically combined forms of chlorine than bacteria found in the unchlorinated Garden Grove system. This suggests that there may be strong selection for chlorine-tolerant microorganisms in chlorinated water distribution systems. (In this regard, Shaffer et al. [31] have shown that poliovirus isolates from finished chlorinated drinking water were considerably more resistant to chlorine than two stock laboratory strains.) Moreover, the differences in the sensitivities of the two bacterial populations does not appear to be related to substances other than chlorine, since the overall water chemistry of the Irvine and Garden Grove systems was very similar (Table 1). (ii) Bacteria from both distribution systems exhibited individual differences in their sensitivities to disinfection by organically combined chlorine in the disk assay. (iii) According to the membrane filtration procedure, gram-positive spore-forming bacilli and some micrococci were the predominant chlorine-resistant microbial

genera detected in the chlorinated Irvine distribution system, whereas gram-positive spore-forming bacilli and actinomycetes were the most common resistant forms in the unchlorinated Garden Grove system. (iv) Finally, filamentous bacteria such as actinomycetes, as well as bacteria attached to suspended particulate matter or otherwise aggregated together in clumps too large to pass through a 2.0-μm Nuclepore membrane filter, exhibited greater resistance to disinfection by free chlorine than unassociated microorganisms.

The occurrence of bacterially colonized suspended particulate matter in the Garden Grove water distribution system has been previously reported (29) and the chemical composition and numbers of these particles in water samples described (B. H. Olson, H. F. Ridgway, E. G. Means, and D. L. Johnson, submitted for publication).

Such bacteria-laden particles are evidently common in raw water sources (12, 15, 23, 25) and in certain municipal drinking water supplies (29) and their existence could account in part for the higher chlorine resistance of the bacteria retained on the 2.0-μm Nuclepore membrane filters. Microbial adhesion to solid surfaces is frequently mediated by extracellular mucopolysaccharides (23), the manufacture and secretion of which by certain strains of *P. aeruginosa* has been shown to dramatically enhance resistance to disinfection by combined chlorine in swimming pool waters (30). The extracellular material observed on the surfaces of the particle-associated cells in this study may perform a similar protective function.

Bacterial colonization of the surfaces of drinking water distribution pipes has been recently documented by the use of scanning electron microscopy (1, 28, 30). Studies in this laboratory have demonstrated that such microbial colonization can occur even in highly chlorinated water systems (>1.0 mg of applied chlorine per liter). The cells comprising many of these microcolonies are frequently covered with extracellular mucopolysaccharide or glycoprotein polymers, which may contribute to their ability to survive and proliferate in such chlorinated environments.

Chlorination has been the most widely practiced method of disinfection for potable waters since the turn of the century and the principal means by which the microbial quality of water is maintained in the United States. However, current public health standards for drinking water based on the coliform index fail to accurately predict large numbers of secondary opportunistic pathogens which these systems can sometimes harbor. In the environment, such bacteria apparently do not display uniform sensitivities to free and combined chlorine, as suggested by the present study and other related investigations (13, 14). Additional research will be required to more accurately assess the chlorine resistance patterns of specific coliform and noncoliform bacterial populations as they exist in various raw and finished waters. Used in conjunction with appropriate selective media, the membrane filtration assay procedure described here should greatly facilitate such studies.

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LITERATURE CITED

1. **Allen, M., R. Taylor, and E. E. Geldreich.** 1980. The occurrence of microorganisms in water main encrustations. Am. Water Works Assoc. J. **72**:614–625.
1a. **American Public Health Association.** 1971. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, Inc., New York.
2. **Azam, F., and R. E. Hodson.** 1977. Size distribution and activity of marine microheterotrophs. Limnol. Oceanogr. **22**:492–501.
3. **Bates, R. C., P. T. B. Shaffer, and S. M. Sutherland.** 1977. Development of poliovirus having increased resistance to chlorine inactivation. Appl. Environ. Microbiol. **34**:849–853.
4. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotic susceptibility testing by a standardized single disk method. Tech. Bull. Regist. Med. Technol. **36**:493–496.
5. **Benarde, M. A., W. B. Snow, V. P. Olivieri, and B. Davidson.** 1967. Kinetics and mechanism of bacterial disinfection by chlorine dioxide. Appl. Microbiol. **15**:257–265.
6. **R. E. Buchanan, and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
7. **Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. **29**:186–194.
8. **Camper, A. K., and G. A. McFeters.** 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. **37**:633–641.
9. **Characklis, W. G., M. G. Trulear, N. Stathopoulos, and L. Chang.** 1980. Oxidation and destruction of microbial films, p. 349–368. *In* L. R. Jolley, W. A. Brungs, and R. B. Cumming (ed.), Water chlorination—environmental impact and health effects, vol. 3. Ann Arbor Science, Ann Arbor, Michigan.
10. **Dennis, W. H., V. P. Olivieri, and C. W. Kruse.** 1979. Mechanism of disinfection: incorporation of C1-36 into f2 virus. Water Res. **13**:363–369.
11. **Dennis, W. H., V. P. Olivieri, and C. W. Kruse.** 1979. The reaction of nucleotides with aqueous hypochlorous acid. Water Res. **13**:357–362.
12. **Geesey, G. G., and J. W. Costerton.** 1979. Microbiology of a northern river: bacterial distribution and relationship

to suspended sediment and organic carbon. Can. J. Microbiol. **25**:1058–1062.
13. **Haas, C. N., and R. S. Engelbrecht.** 1980. Physiological alterations of vegetative microorganisms resulting from chlorination. J. Water Pollut. Control Fed. **52**:1976–1989.
14. **Haas, C. N., and J. P. Gould.** 1979. Disinfection. J. Water Pollut. Control Fed. **51**:1232–1242.
15. **Harvey, R. W., and L. Y. Young.** 1980. Enrichment and association of bacteria and particulates in salt marsh surface water. Appl. Environ. Microbiol. **39**:894–899.
16. **Hoadley, A. W., and J. P. Gould.** 1977. Disinfection. J. Water Pollut Control Fed. **49**:1067–1073.
17. **Kalakoutskii, L. V., and L. M. Pouzharitskaja.** 1973. The Streptomyces spore: its distinct features and geroninal behaviour, p. 155–178. *In* G. Sykes and F. A. Skinner (ed.), Actinomycetales: characteristics and practical importance. Academic Press Inc. London.
18. **Knox, W. E., P. K. Stumpf, D. E. Green, and Y. H. Auerbach.** 1948. The inhibition of sulfhydryl enzymes as the basis of the bacterial action of chlorine. J. Bacteriol. **55**:451–458.
19. **Larson, R. A., and A. L. Rockwell.** 1979. Chloroform and chlorophenol production by decarboxylation of natural acids during aqueous chlorination. Environ. Sci. Technol. **13**:325–329.
20. **Lassen, J.** 1975. Rapid identification of gram-negative rods using a three-tube method combined with a dichotomic key. Acta Pathol. Microbiol. Scand. Sect. B **83**:525–533.
21. **LeChevallier, M. W., R. J. Seidler, and T. M. Evans.** 1980. Enumeration and characterization of standard plate count bacteria in chlorinated and raw water supplies. Appl. Environ. Microbiol. **40**:922–930.
22. **Ludovici, P. P., R. A. Phillips, and W. S. Jeter.** 1977. Comparative inactivation of bacteria and viruses in tertiary-treated wastewater by chlorination, p. 359–390. *In* J. D. Johnson (ed.), Disinfection: water and wastewater. Ann Arbor Science, Ann Arbor, Michigan.
23. **Marshall, K. C.** 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge, Massachusetts.
24. **Means, E. G., L. Hanami, H. F. Ridgway, and B. H. Olson.** 1981. Enumeration of bacteria in potable water distribution systems: evaluation of media and plating techniques. Am. Water Works Assoc. J. **53**:585–590.
25. **Paerl, H. W.** 1973. Detritus in Lake Tahoe: Structural modification by attached microflora. Science **180**:496–498.
26. **Palin, A. T.** 1977. Water disinfection—chemical aspects and analytical control, p. 67–89. *In* J. D. Johnson (ed.), Disinfection: water and wastewater. Ann Arbor Science, Ann Arbor, Michigan.
27. **Pereira, W. E., Y. Hoyano, R. E. Summons, V. A. Bacon, and A. M. Duffield.** 1973. Chlorination studies 2: reaction of aqueous hypochlorous acid with alpha amino acids and dipeptides. Biochim. Biophys. Acta **313**:170–180.
28. **Ridgway, H. F., E. G. Means, and B. H. Olson.** 1981. Iron bacteria in drinking-water distribution systems: elemental analysis of *Gallionella* stalks, using X-ray energy-dispersive microanalysis. Appl. Environ. Microbiol. **41**:288–297.
29. **Ridgway, H. F., and B. H. Olson.** 1981. Scanning electron microscope evidence for bacterial colonization of a drinking-water distribution system. Appl. Environ. Microbiol. **41**:274–287.
30. **Seyfried, P. L., and D. J. Fraser.** 1980. Persistence of *Pseudomonas aeruginosa* in chlorinated swimming pools. Can. J. Microbiol. **26**:350–355.
31. **Shaffer, P. T. B., T. G. Metcalf, and O. J. Sproul.** 1980. Chlorine resistance of poliovirus isolants recovered from drinking water. Appl. Environ. Microbiol. **40**:1115–1121.
32. **Shih, K. L., and J. Lederberg.** 1976. Chloramine mutagenesis in *Bacillus subtilis*. Science **192**:1141.
33. **Shih, K. L., and J. Lederberg.** 1976. Effects of chloramine on *Bacillus subtilis* deoxyribonucleic acid. J. Bacteriol. **125**:934–945.
34. **Stagg, C. H., C. Wallis, and C. H. Ward.** Inactivation of clay-associated bacteriophage MS-2 by chlorine. Appl. Environ. Microbiol. **33**:385–391.
35. **Wenkobachar, C., L. Iyengar, and A. V. S. P. Rav.** 1975. Mechanism of disinfection. Water Res. **9**:119–124.
36. **Wenkobachar, C., L. Iyengar, and A. V. S. P. Rav.** 1977. Mechanism of disinfection: effect of chlorine on cell membrane function. Water Res. **11**:727–729.